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The chick limb: embryology, genetics and teratology

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ABSTRACT The chick embryo has a long history in investigations of vertebrate limb development because of the ease with which its limbs can be experimentally manipulated. Early studies elucidated the fundamental embryology of the limb and identified the key signalling regions that govern its development. The chick limb became a leading model for exploring the concept of positional information and understanding how patterns of differentiated cells and tissues develop in vertebrate embryos. When developmentally important molecules began to be identified, experiments in chick limbs were crucial for bridging embryology and molecular biology. The embryological mechanisms and molecular basis of limb development are largely conserved in mammals, including humans, and uncovering these molecular networks provides links to clinical genetics. We emphasise the important contributions of naturally occurring chick mutants to elucidating limb embryology and identifying novel developmentally important genes. In addition, we consider how the chick limb has been used to study mechanisms involved in teratogenesis with a focus on thalidomide. These studies on chick embryos have given insights into how limb defects can be caused by both genetic changes and chemical insults and therefore are of great medical significance.

KEY WORDS: *pattern formation, signalling, talpid, disease model, thalidomide*

Introduction

The chick embryo has a proud history as a premier model for studying vertebrate limb development. The developmental stages and morphology of chick limbs conform to the typical vertebrate plan except that there are only three digits in the wing and four in the leg (Fig. 1). The chick limb came to prominence in the mid-20th century largely because of pioneering embryological experiments by John Saunders which identified key signalling centres involved in its development (Saunders, 1948; Saunders and Gasseling, 1968; reviewed Tickle, 2017). Lewis Wolpert and colleagues then used the chick limb to explore the role of signalling centres in specifying positional information in pattern formation- the developmental process that generates spatially organized arrays of differentiated cells and tissues (Wolpert, 1969). For details about why Wolpert decided to work on the limb see his contribution to this issue (Wolpert, 2018). The adoption of the chick limb as a model for pattern formation meant that it came to have a much wider significance, elucidating general principles that apply to other regions of the embryo. Experiments on the chick limb also showed that it is capable of self-organization and can generate a periodic pattern

which is a feature of many biological systems.

The ease with which developing limbs can be manipulated in chick embryos provided the basis for uncovering the fundamental embryology. Classical manipulations include ablating parts of the limb, transplanting tissues to different positions, separating tissues and then recombining them, making tissue chimeras and fate maps. When developmentally important genes began to be discovered, experiments on chick embryos were crucial for bridging experimental embryology with molecular biology. Methods were devised to manipulate signalling pathways by grafting cells producing signalling molecules and implanting beads soaked in various chemicals. The use of beads, first pioneered in chick wing development (Eichele *et al.*, 1984), has been adopted widely by developmental biologists to apply not only signalling molecules locally to embryos and organ cultures but also other chemicals such as small molecule inhibitors. Transient transgenesis in which gene expression constructs are delivered to developing chick limbs using retroviruses (Morgan *et al.*, 1992) or by electroporation was

Abbreviations used in this paper: FGF, fibroblast growth factor; GFP, green fluorescent protein; Shh, sonic hedgehog; ZRS, zone of polarizing activity regulatory sequence.

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also introduced complementing emerging genetic approaches in the mouse. In the last 10 years or so, the creation of genomic resources and sequencing of the chicken genome have ensured that the chick remains at the forefront of limb research. In addition, transgenic chickens such as those expressing Green Fluorescent Protein (GFP) became available which have further enhanced chimeric limb analysis.

Naturally occurring chicken mutants have provided another route to understanding limb embryology and led to the discovery of novel developmental genes. Because generation of mouse limb mutants is now relatively routine, it is easy to overlook how valuable naturally occurring limb mutants are. Many classical recessive embryonic lethal chicken mutants, identified because of their failure to hatch, such as the *talpid* mutants, have abnormal limbs. Early researchers used these chicken mutants to illuminate limb embryology. And of course, nature frequently designs better experiments than scientists, creating novel phenotypes which can lead to unexpected avenues of understanding and identifying genes which might otherwise have been missed in mammalian systems.

Studies on the chicken embryo have been especially impactful because even though the morphology of the limbs of birds and mammals has diverged during evolution, the developmental mechanisms are generally conserved, including in humans. Furthermore, understanding chick limb development at the genetic level has provided direct links with clinical genetics and the genes responsible for limb abnormalities in patients (reviewed Zuniga *et al.*, 2012). We will emphasise the clinical relevance of embryological and molecular studies on limb development in both normal and mutant chick embryos. In addition, although developmental biologists have generally focussed on genetic changes that cause limb abnormalities, exposure to harmful chemicals during embryonic development is another important cause. The chick embryo

offers a fantastic model system to study how chemical teratogens produce limb defects *live* and *in-vivo* as it can be readily treated by injections into the yolk or by dropping solutions on to it. Romanoff (1972) lists over 100 inorganic and organic chemicals that have been applied to chick embryos and about a third of these were reported to affect limb development. Here we will concentrate on thalidomide, an infamous limb teratogen with a long history of being investigated in the chick embryo.

Contributions of the chick to understanding limb embryology

Limb initiation and cell lineages

Chick limbs develop from small buds that arise at appropriate levels along the main body axis (wing buds opposite somites 15-20, leg buds opposite somites 26-31). Transplantation experiments in chick embryos were instrumental in showing that limb-forming regions and their antero-posterior polarity are determined long before any limb buds are visible. Further cut and paste experiments indicated that signals from neighbouring tissues are involved in specifying the position of the limb-forming regions at the sides of the body (reviewed Tickle, 2015).

Experiments on chick embryos revealed the origins of the cells that make up the limb buds. The early limb bud consists of a vascularised core of undifferentiated mesenchyme cells encased in ectoderm with a thickened ectoderm around the rim of the bud known as the apical ectodermal ridge (apical ridge; Saunders, 1948). Chick-quail chimeras demonstrated that cells from adjacent somites migrate into the limb-forming regions and give rise to the myogenic cells of the muscles (Christ *et al.*, 1977) and to the vasculature (Ambler *et al.*, 2001). Likewise, chick-mouse chimeras demonstrated that mouse somitic cells can give rise to

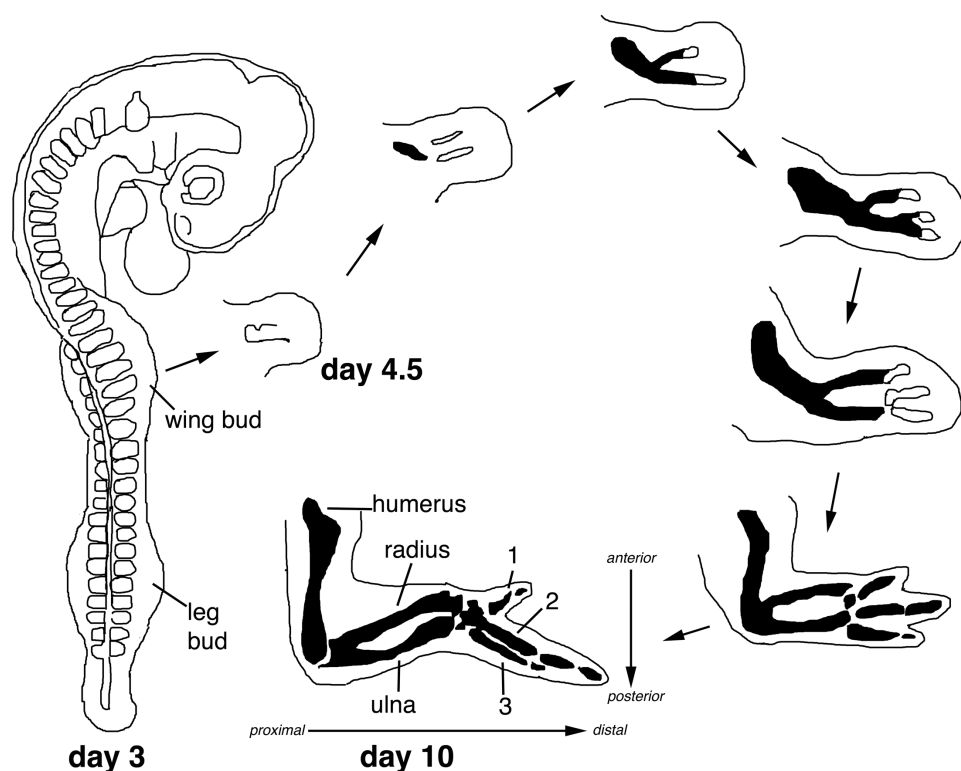


Fig. 1. Vertebrate limb development illustrated by the chick wing. Small protrusion from the flank forms bud, bud grows distally eventually acquiring characteristic wing shape. Skeletal development represented by condensations of chondrogenic cells (outlined within bud), cartilage differentiation (black). Skeletal elements laid down in sequence along proximo-distal axis starting with humerus, accompanied by splitting of dorsal and ventral muscle masses to form extensors and flexors respectively (not illustrated). Series of condensations, giving rise to digits, form within hand-plate, digits then separated by programmed cell death in interdigital regions. Each digit morphologically distinct in a precise pattern across antero-posterior axis, digit 1 anterior; digit 3 posterior (digits previously designated 234). Nerves grow into limb after pattern of skeleton and musculature established. Dorsal uppermost. Days = days after laying. Re-drawn after Tickle and Eichele (1994).

limb myoblasts and angioblasts. The lateral plate mesoderm gives rise to the connective tissues (Pearse *et al.*, 2007). Observations on chick embryos have shown that the lateral plate mesoderm in the limb-forming regions is augmented by cells from the coelomic epithelium that have undergone an epithelial-mesenchymal transition (Gros *et al.*, 2014). The origin of the limb bud ectoderm has been traced in chick embryos using either chick-quail chimeras or the lipophilic dye, Dil, to label small groups of cells (Michaud *et al.*, 1997; Altabef *et al.*, 1997). The latter technique showed that the limb bud ectoderm consists of cell-lineage restricted dorsal and ventral compartments; ectoderm cells that will form the apical ridge are initially scattered and then migrate to the compartment boundary.

The apical ectodermal ridge and limb bud outgrowth

The apical ridge is required for outgrowth and laying down of the pattern of structures along the proximo-distal axis. Experiments by Saunders in the 1940's showed that when the apical ridge is surgically removed from chick wing buds, outgrowth ceases and truncated wings develop (Saunders, 1948; see also Summerbell, 1974). The level of truncation depends on the stage at which the apical ridge is removed, more severe truncations being produced when the ridge is removed at earlier stages. In contrast, an apical ridge grafted to the dorsal surface of a wing bud signals to the underlying mesenchyme inducing a new outgrowth (Saunders and Gasseling, 1968). Several chicken mutants have wings truncated at the shoulder girdle; a *limbless* mutant and four independent *wingless* mutant strains (Zwilling, 1974; Carrington and Fallon, 1984; Ohuchi *et al.*, 1997a; Hinchliffe and Ede, 1973). Mesenchymal-ectodermal recombination experiments with *wingless* mutants have shown that the defect lies in the ectoderm (e.g. Hinchliffe and Ede, 1977) confirming its crucial role in limb bud outgrowth first proposed by Saunders. Recombination experiments in which the apical ridge is exchanged between chick wing bud and chick leg bud and between chick wing bud and mouse fore-limb bud showed that its function is highly conserved between limb buds and between birds and mammals. Thus, congenital limb truncations in human patients could be caused by failure of apical ridge signalling.

Proximo-distal patterning and relevance to limb deficiencies

The current view of how the proximo-distal pattern of the vertebrate limb is specified comes almost exclusively from chick experiments. There has been a long-running debate about whether proximo-distal positional information is specified by the length of time that cells spend in a progress zone, a region of undifferentiated proliferating cells at the tip of the bud (Summerbell *et al.*, 1973) or are pre-specified in the early bud (Dudley *et al.*, 2002). In the former model, the apical ridge acts permissively to maintain the progress zone, consistent with normal wings developing after exchanging the apical ridge between wing buds at different developmental stages (reviewed Saunders, 1977). Recent experiments in which the long term fate of transplanted mesenchyme cells was followed using tissue from the GFP transgenic chicken demonstrated an intrinsic timing mechanism (Saiz-Lopez *et al.*, 2015; Saiz-Lopez *et al.*, 2017), while grafting experiments by others suggested that proximal positional information is specified in the early wing bud by diffusible signals from the body wall (Cooper *et al.*, 2011; Rosello-Diez *et al.*, 2011).

The mechanisms that specify proximo-distal pattern have implications for understanding the basis not only of distal deficiencies

such as truncations but also phocomelia, a transverse deficiency in which proximal structures are absent or severely shortened, but distal structures relatively unaffected. The very early chick wing bud can recover after removing most of the mesenchyme provided the apical ridge is intact (Mahony and Vargesson, 2013). At slightly later stages, mesenchyme removal (Mahony and Vargesson, 2013) and X-irradiation and treatments with chemicals such as nitrogen mustard, which kill mesenchyme cells, lead to phocomelia. According to the progress zone model, the sparing of distal structures would be due to cells spending longer in the progress zone in order to replace missing cells, thus becoming distalized (Wolpert *et al.*, 1979). An alternative interpretation is that proximal cell populations are selectively eliminated (Galloway *et al.*, 2009). Both truncated limbs and phocomelia are seen in patients whose mothers took thalidomide at a critical period during pregnancy and experiments on chick embryos have contributed to understanding how thalidomide causes these defects.

Studies in the chick on limb deficiencies produced by thalidomide

The chick embryo was one of the first models used to investigate the mechanisms of thalidomide action. Perhaps surprisingly, thalidomide has little effect on rodent embryos, so research on its actions has necessarily been carried out on other organisms (Vargesson, 2013). Initial studies indicated that thalidomide is harmful to early chick embryos and injection into the yolk caused facial and spinal anomalies and shortening and stunting of the legs (Boylen *et al.*, 1963). In another study, thalidomide was applied directly over the wing bud and this damaged and dilated the axial artery supplying it (Jurand, 1966). It was not until seminal work by Judah Folkman and colleagues (D'Amato *et al.*, 1994), using rodent angiogenic cornea assays, however that it was proved that thalidomide is anti-angiogenic. This discovery suggested that the teratogenic effects of thalidomide on limb development are due to these inhibitory effects on angiogenesis. Radiography of thalidomide survivors indicating altered nerve patterns and some rabbit embryo work showing loss of nerves in thalidomide damaged limbs had led to an alternative theory that thalidomide targets neural crest and nerves (McCredie and McBride, 1973). Studies on chick embryos showed that experimentally inhibiting innervation of developing limbs did not produce defects although they were shorter (Strecker and Stephens, 1983; Mahony *et al.*, In press) so nerves are more likely to be affected secondarily.

Structural analogs of thalidomide which have either anti-inflammatory actions or anti-angiogenic actions have been screened in chick embryos. Anti-inflammatory thalidomide analogs and other metabolic by-products of the drug did not cause any developmental defects. But the antiangiogenic analog, CPS49, caused a range of wing defects including truncations and phocomelia, the precise defect depending on the stage at which the embryos were treated (Therapontos *et al.*, 2009; Fig. 2). A crucial observation was that blood vessel damage was detected within a couple of hours of treatment resulting in large areas of the wing bud becoming avascular and this was followed by cell death several hours later. Interestingly, only newly formed or forming vessels were destroyed, whereas those no longer undergoing angiogenesis which possessed smooth muscle coats were unharmed. This could explain why thalidomide appears to have specific effects on the limbs as these are the main organs at these stages in which angiogenesis

is occurring (Vargesson and Laufer, 2001). The key importance of thalidomide-effects on blood vessels was further emphasised by finding that molecules which protect blood vessels, such as nitric oxide, prevent thalidomide-induced limb defects in chick embryos (Siamwala *et al.*, 2012).

These observations on chick embryos indicate that the anti-angiogenic action of thalidomide could cause its teratogenic effects on human limb development. But questions remain about how damaged blood vessels lead to the specific limb defects such as phocomelia (Tabin, 1998). As hypothesised for the effects of X-irradiation on the chick wing (Wolpert *et al.*, 1979), loss of newly formed or forming vessels in the developing limb could result in mesenchymal cell death in the progress zone resulting in distalization of remaining cells. Anti-angiogenic, anti-cancer drugs, Sunitinib and Sorafenib, both antagonists of the signalling pathway involved in angiogenesis, also result in limb abnormalities when applied to chick embryos (Beedie *et al.*, 2016a). This suggests that all anti-angiogenic drugs need to be carefully regulated to ensure they are not taken by pregnant women.

The polarizing region and antero-posterior pattern formation

Experiments carried out on chick wing buds by Saunders led to the discovery of the zone of polarizing activity (ZPA; polarizing region), the small region of mesenchyme cells at the posterior margin of the early limb bud which specifies antero-posterior pattern (Saunders and Gasseling, 1968). When the polarizing region from a chick wing bud was grafted to the anterior margin of another wing bud, up to six digits developed instead of three, with additional digits arising from the anterior part of the bud in mirror-image symmetry with the normal set; the pattern of the fore-arm including the muscle pattern can also be duplicated (Shellswell

and Wolpert, 1977).

A long-standing model for polarizing signalling in the chick wing bud is based on the concept of positional information (Wolpert, 1969). It was proposed that the polarizing region secretes a morphogen that spreads across the bud and establishes a concentration gradient with cells being informed of their position by the local morphogen concentration; cells then interpret this information to form the appropriate structure. Positional information is established in the early wing bud and then remembered. A wealth of experiments supports this model but also revealed that timing and direct effects on growth are involved (reviewed Towers and Tickle, 2009). It had also been known for a long time that chick limb mesenchyme is able to self-organize. When the mesenchymal core of chick limb buds is disaggregated into single cells, then reaggregated and placed back inside an ectodermal jacket, such “recombinant limb buds” give rise to a series of repeated digit-like structures (reviewed Saunders, 1977). However, when a polarizing region is grafted to one edge of a “recombinant limb”, the antero-posterior digit pattern is re-established. Thus, self-organization, postulated to occur via a Turing-type mechanism, might co-operate with positional information (Wolpert, 1989; Green and Sharpe, 2015; Pickering and Towers 2016). Other experiments on developing chick legs showed that digit morphology can be altered at the foot-plate stage by grafting mesenchyme from one interdigital space to another (Dahn and Fallon, 2000) indicating that further signalling interactions translate positional information into final digit anatomy.

Polarizing activity has been detected when tissue from the posterior margin of mammalian limb buds, including human limb buds, is grafted to the anterior margin of chick wing buds resulting in induction of additional digits (Fallon and Crosby, 1977). These experiments show that polarizing region signalling is highly

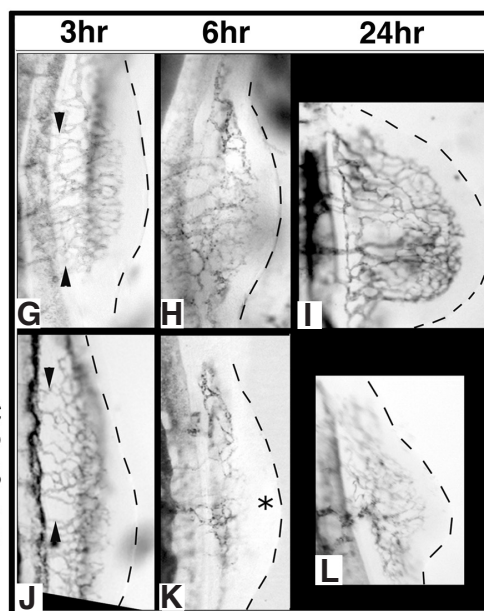
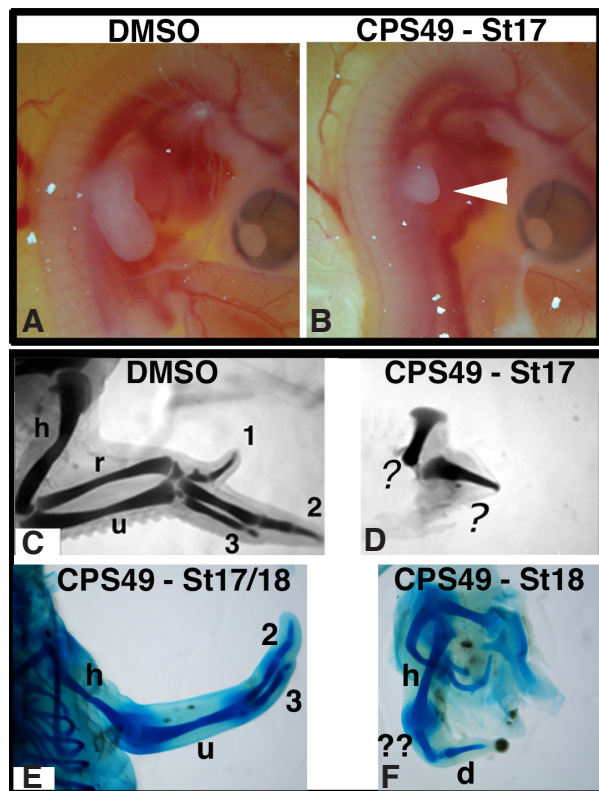


Fig. 2. Anti-angiogenic thalidomide analog, CPS49, causes chick wing malformations. DMSO treatments are controls. (A,B) Living embryos 48hrs after treatment showing stunting of wing bud out-growth by CPS49 (B, white arrowhead). (C-F) Cartilage stains of 10 day wings. Normal wing skeleton, control (C); range of truncations and reduction defects following CPS49 treatments (D-F). (G-L) Vasculature visualized by Indian ink injections. CPS49 inhibits wing vessel angiogenesis within 3hrs (G arrowheads, compare with control); at 6 hrs, large avascular zone in distal wing bud (K, asterisk compare with H); at 24hrs wing bud severely stunted with markedly reduced vasculature (L compare with I, bud with highly patterned and intricate vascular network). Abbreviations: St, Hamburger-Hamilton stage treatment; h, humerus; u, ulna; r, radius; 1, digit1; 2, digit2; 3, digit3; d, unidentified digit; ?, two severely truncated articulating cartilage elements; ??, shortened ulna. (A-D, G-L) Modified from Therapontos *et al.*, (2009). (E,F) Images from N. Vargesson (University of Aberdeen, Scotland).

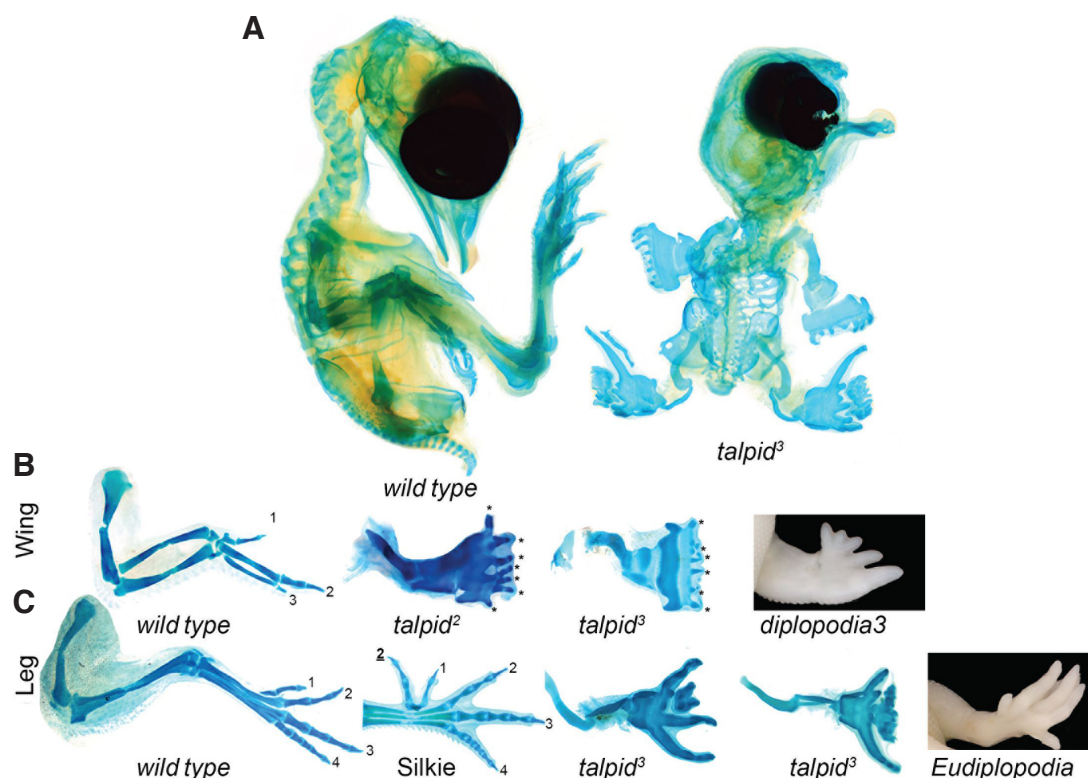


Fig. 3. Chicken mutants. (A) Cartilage skeletons of E14 *talpid³* mutant and normal sibling, showing polysyndactylous wings and legs in mutant. Note other malformations - 'peg' like lower jaw, holoprosencephaly, loss of upper jaw. **(B)** Comparison of normal wing, *talpid²* wing (Samantha A. Brugmann, Cincinnati Children's Hospital), *talpid³* wing and *diplopodia³* wing (E.A. O'Hare and M.E. Delany, UC Davis); all polysyndactylous but with variations. *dp³* maintains digit identity (not shown) whereas *talpid²* and *talpid³* do not. **(C)** Comparison of normal leg with digits 1, 2, 3, 4 (from anterior to posterior), Silkie leg with preaxial polydactyly (digits 2, 1, 2, 3, 4), two *talpid³* legs showing variable polysyndactyly and *Eudiplopodia* leg (E.A. O'Hare and M.E. Delany, UC Davis) with polydactyly and additional digits arising dorsally and ventrally.

conserved and that digit anatomy depends on the interpretation of positional information as the additional digits induced are chick wing digits. In the chick leg, the most posterior digit comes from the polarizing region itself and in the mouse limb, the two most posterior digits (reviewed Tickle and Towers, 2017). It has been suggested that these posterior digits are specified by a timing mechanism whereas the anterior digits are specified, as in the chick wing, by graded signalling. The implication of these studies is that pre-axial polydactyly in human patients -additional digits anteriorly -could be caused by having polarizing activity at both anterior and posterior margins of the limb bud.

The polarizing region and maintenance of the apical ectodermal ridge

The polarizing region has a pivotal role not only for specifying the antero-posterior pattern of structures that develop distal to the elbow/knee but also maintaining the apical ridge over the adjacent posterior part of the bud. The antero-posterior extent of the apical ridge determines the width of bud outgrowth and, consistent with a Turing-type mechanism, the number of digits is determined by bud width. That the distal structures arise from the posterior part of the wing bud has been shown by generations of fate maps through marking mesoderm cells with carbon particles, chick-quail chimeras, Dil labelling, and confirmed by grafts from the GFP chicken (reviewed Towers and Tickle, 2009; Towers *et al.*, 2011). It was proposed that the polarizing region controls production of a factor - apical ectoderm maintenance factor - which maintains the apical ridge (reviewed Saunders, 1977). This dependence of the apical ridge on the polarizing region signalling means that limb truncations could arise indirectly because of defective polarizing region signalling. It was also shown that polarizing region grafts

have to be grafted adjacent to the ridge in order to induce additional digits suggesting that the polarizing region is maintained by the apical ridge.

Polarizing activity in *talpid* polydactylous chicken mutants

Talpid² (Abbott *et al.*, 1960) and *talpid³* (Ede and Kelly, 1964) both have polysyndactyly - limbs with many fused digits (Fig. 3). The formation of many digits in *talpid³* correlates with broadened limb buds and an antero-posteriorly extended apical ridge. Donald Ede's work on *talpid³* was ahead of its time and culminated in the first mathematical computer model of limb bud growth. The model could simulate the broadened *talpid³* limb bud based on unrestricted growth across the antero-posterior axis (Wilby and Ede, 1975). Mesenchymal-ectodermal recombination experiments with *talpid³* wing buds revealed that the defect lies in the mesenchyme and that dissociated *talpid³* limb mesenchyme cells could form digits spontaneously. Moreover, grafting the polarizing region from *talpid³* wing buds to normal wing buds showed that the *talpid³* mutation caused a failure of the *talpid³* cells to receive, but not transmit the polarizing signal (Ede and Shamsahidjani, 1983).

Ectoderm and dorso-ventral pattern formation

Chick experiments showed that the ectoderm covering the sides of the bud controls the dorso-ventral pattern (reviewed Saunders, 1977). In mesenchymal-ectodermal recombinations, in which the dorso-ventral axis was reversed relative to the mesenchyme, the polarity of distal structures conformed to the polarity of the ectoderm. The importance of ectodermal signals in controlling dorso-ventral pattern was further demonstrated by the "double -dorsal" muscle pattern of supernumerary wing bud tips - induced by grafting an apical ridge to the dorsal surface of a wing bud - which were covered

on both sides by dorsal ectoderm (Shellswell and Wolpert, 1977). A “double-dorsal” pattern indicated by conical nails was also observed in two toes arising from the dorsal surface of a polydactylous human limb (D’Souza *et al.*, 1998) suggesting that the ectoderm controls dorso-ventral pattern in human limb development.

Insights into the molecular basis of limb development from the chick

Apical ectodermal ridge signalling

Studies on the mouse limb showed that the apical ectodermal ridge expresses *Fgf4* and *Fgf8* and a key experiment in the chick wing showed that a bead soaked in an FGF protein could substitute for an extirpated apical ectodermal ridge (Niswander *et al.*, 1993). In the Japanese *wingless* mutant, *Fgf4* is expressed in the apical ectodermal ridge at first but later lost. Just as an FGF4 bead rescues normal wing development after apical ridge removal, the Japanese *wingless* wing can similarly be rescued by implanting an FGF bead, thus showing that the *wingless* defect lies in failure to maintain *Fgf4* signalling in the apical ridge (Ohuchi *et al.*, 1997a). The fact that only wings are absent in the mutant points to an unexpected difference in apical ridge maintenance in wing versus leg.

Even more strikingly, FGFs, applied on beads to the interlimb flank of a chick embryo, induce ectopic limbs (Cohn *et al.*, 1995). This finding contributed to uncovering the role in limb initiation for mesenchymal FGFs (*Fgf10*), which operate in positive feedback loops with FGF and Wnt family signalling ligands produced by the apical ridge (Ohuchi *et al.*, 1997b). Subsequently, it emerged that Wnts are also expressed in the apical ridge and grafts of Wnt-producing cells to the interlimb flank of a chicken embryo also induce ectopic limbs (Kawakami *et al.*, 2001). *Fgf10*, in cooperation with the transcription factor *Tbx5*, is involved in the early delamination of cells of the coelomic epithelium, which contribute to the limb bud mesenchyme. *Tbx5* and its close relative *Tbx4* have striking expression patterns -first shown in chick embryos -with *Tbx5* being expressed specifically in wing buds and *Tbx4* in leg buds. Subsequent work in transgenic mice showed that *Tbx5* is essential for fore-limb development and that *Tbx4* can replace its function suggesting that the genes have equivalent functions in developing limbs (reviewed Nishimoto and Logan, 2016). *Tbx5* mutations are found in patients with Holt-Oram syndrome, characterized by arm and heart defects.

FGFs are expressed in the apical ridge throughout the laying down of the entire proximo-distal pattern. Experiments in the chick leg have shown that regression of the apical ridge and consequent cessation of FGF signalling induces formation of the terminal phalanx of a digit while extending FGF signalling experimentally leads to development of an additional phalanx (Sanz-Ezquerro and Tickle, 2003). Mutations in FGF receptors are clinically important, for example, in patients with Aperts syndrome who have digit anomalies.

As already mentioned, the prevailing model for how proximo-distal positional values are specified involves an intrinsic timing mechanism that operates in cells in a progress zone. However, the specification of proximal structures appears to involve retinoic acid signals from the flank counteracting FGF signals from the apical ridge (Cooper *et al.*, 2011; Rosello-Diaz *et al.*, 2011). As the wing bud elongates away from the flank, the concentration of retinoic acid falls and this starts the timing mechanism (Saiz-Lopez *et al.*, 2015). This role in proximal limb patterning could be coupled with

a role in limb initiation, since retinoic acid receptor antagonists inhibit chick wing development. Recent work also suggests that retinoic acid could be involved in chick leg initiation (reviewed Nishimoto and Logan, 2016). However, these roles for retinoic acid are still debated.

Molecular basis of proximo-distal positional values

It remains unclear how positional information is encoded along the proximo-distal axis. Good candidates include the products encoded by 5’ genes of the *Hoxa* and *Hoxd* clusters with spatially restricted overlapping expression patterns. Detailed analysis in chick limbs led to the suggestion that there are two phases of expression (Nelson *et al.*, 1996) and subsequent work in the mouse has identified two different enhancers on either side of the *Hoxd* cluster (Montavon and Duboule, 2013). Extensive genetic analyses in the mouse limb including the creation of multiple knock-outs suggested that different combinations of *Hox* genes control regional identity along the proximo-distal axis (Wellik and Capecchi, 2003). *Hoxd13* and *Hoxa13* specify the digits and mutations in these genes have been detected in patients with synpolydactyly and hand-foot-genital syndrome respectively.

Other transcription factors may also influence regional identity. The genes encoding Meis1 and Meis 2 transcription factors are expressed in cells that give rise to proximal structures and studies in the chick showed that they are responsive to retinoic acid signalling (Mercader *et al.*, 2000). The *Shox* transcription factor could also contribute to specifying positional identity. *Shox* is expressed in cells in the chick wing bud that give rise to the ulna/radius and is repressed by both retinoic acid and FGF signals (Tiecke *et al.*, 2006). Mutations in *SHOX* have been identified in patients with short stature conditions such as Langer mesomelic syndrome in which the lower arm and lower leg are particularly short and *SHOX* deficiency contributes to Turner syndrome. The mouse does not have a *Shox* gene -only the closely related gene *Shox2* - so the chick provides the experimental model for studying its function. Furthermore an *in vivo* assay in the chick wing bud identified enhancer activity in regions downstream of *SHOX* deleted in short stature patients who have an intact coding region for the gene thus providing an explanation for their condition (Sabherwal *et al.*, 2007).

Polarizing region signalling

The first molecule found to mimic signalling of the polarizing region was retinoic acid and application of retinoic acid to the anterior margin of a chick wing bud resulted in mirror image duplications of the pattern of digits (Tickle *et al.*, 1982). The demonstration that retinoic acid acted in a concentration and time-dependent manner, required of a polarizing region signal, was consistent with it being the endogenous signal. However, further experiments on the chick wing indicated that retinoic acid induced a new polarizing region and that the secreted signalling molecule Sonic hedgehog (*Shh*; Riddle *et al.*, 1993) is the polarizing region morphogen.

Shh expression coincides with maps of polarizing region activity at the posterior margin of the chick wing bud and *Shh* protein can duplicate the pattern of chick wing digits when provided by transfected cells or on beads grafted to the anterior margin of the bud. The crucial role for *Shh* was further revealed by the loss of digits, in chick limbs, when cyclopamine was applied to inhibit Smoothened (*Smo*), the transmembrane protein that activates the

Shh intracellular transduction pathway, and in mouse limbs, when *Shh* was functionally inactivated (reviewed Tickle and Towers, 2017). Furthermore, in mouse mutants with preaxial polydactyly, such as *Sasquatch*, *Shh* is expressed both anteriorly and posteriorly in the limb buds. *Sasquatch* is an insertional mouse mutant and particularly informative as ectopic *Shh* expression in the limb bud is caused by the transgene inserting into a long range regulatory sequence that controls *Shh* expression specifically in the limb, now known as the Zone of Polarizing activity Regulatory Sequence (ZRS; reviewed Hill and Lettice, 2013). Chromosomal breakpoints or mutations in the ZRS are associated with pre-axial polydactylous conditions in human patients as well as in cats and dogs. Deletion of the entire ZRS region in mice leads to loss of digits and mutations in this region occur in the human condition *Acheiropodia* in which structures distal to the elbow and knee are missing.

Chicken breeds and mutants with ZRS-associated mutations

Many Asian chicken breeds, such as the Silkie, with extra toes - pre-axial polydactyly (Fig. 3) - have a dominant point mutation in the ZRS resulting in ectopic *Shh* expression at the anterior of the leg bud (Dunn *et al.*, 2011, Maas *et al.*, 2012). The polydactylous locus in European polydactylous breeds derived from the Dorking breed also maps to an area containing the ZRS but is not the same mutation (Zhang *et al.*, 2016). It has been generally concluded from these observations that the ZRS sequence inhibits *Shh* expression in the anterior of the limb bud but genetic crosses between polydactylous Silkie birds and non-polydactylous revertants with a normal ZRS sequence showed that the ZRS also controls the level of Shh signalling in the posterior (Johnson *et al.*, 2014).

In contrast to these polydactylous breeds, the recessive *oligozeugodactyly* chicken mutant (*ozd*) lacks the ulna and all the digits in the wing and the fibula and digits 2-4 in the leg (Symth *et al.*, 2000) reminiscent of the limb of *Shh* mouse mutants. John Fallon and collaborators showed that *Shh* expression is absent specifically in the limb buds but that adding recombinant Shh protein to the *ozd* wing buds restored the normal pattern, consistent with the *ozd* mutation affecting *Shh* expression itself, rather than disrupting downstream signalling (Ros *et al.*, 2003). Mapping of the *ozd* mutation revealed a large 1654bp deletion partially overlapping the ZRS (Maas *et al.*, 2012) thus explaining the lack of limb bud *Shh* expression.

Insights into mechanisms of Shh signalling from the chick

Extensive experimental analyses on the chick wing have defined the parameters by which graded Shh signalling specifies antero-posterior positional values and directly stimulates mesenchyme proliferation (Towers *et al.*, 2008). In addition, a key finding from experiments on chick limb buds is that Fibroblast Growth Factor 4 (*Fgf4*) signalling by the apical ridge maintains *Shh* expression in the polarizing region, while, in turn, Shh signalling maintains *Fgf4* expression in the apical ridge, thus establishing a positive feedback loop that maintains bud outgrowth (Niswander *et al.*,

1994, Laufer *et al.*, 1994). Work on the mouse identified the BMP (Bone Morphogenetic Protein) antagonist, Gremlin1, as the apical ectoderm maintenance factor postulated by earlier grafting experiments on the chick wing.

A gradient of Shh has been demonstrated across the antero-posterior axis of chick wing buds (Zeng *et al.*, 2001) but it is unclear whether this occurs by free diffusion and/or by active transport between cells in specialised structures called filopodia which have been studied in chick limb mesenchyme cells (Sanders *et al.*, 2013). Mathematical modelling of digit specification in the chick leg is consistent with the polarizing region producing a digit in response to the timing of Shh signalling (Woolley *et al.*, 2014). In the chick wing, the duration of *Shh* expression in the polarizing region is controlled by a timing mechanism, initially set by the level of retinoic signalling (Chinnaiya *et al.*, 2014). Thus, as with proximo-distal patterning, antero-posterior patterning can involve both graded signalling and time-based mechanisms.

What is the basis by which graded Shh signalling specifies antero-posterior positional values? Genetic analyses in the mouse demonstrated that the transcriptional effectors of Shh signalling are the Gli transcription factors, (Gli1, 2 and 3). Gli3 plays the predominant role in the developing limb and functions primarily as a transcriptional repressor. Shh prevents the processing of the full-length Gli3 protein into a repressor form and a gradient of Gli3A/Gli3R from posterior to anterior has been demonstrated in the chick wing and the mouse limb (reviewed Tickle and Towers, 2017). Mutations in Gli3 are associated with human congenital limb abnormalities.

Chicken talpid mutants and ciliopathy genes

When Shh was identified as the molecule primarily responsible for signalling of the polarising region, it was initially surprising that *Shh* expression itself appeared to be localised normally to the posterior margin of the polydactylous *talpid*^l limb buds. However, *Ptch1*, which encodes the Shh receptor and is a direct target of Shh signalling, was not expressed at high levels (Lewis *et al.*, 1999). Thus, the *talpid*^l defect lay, as Ede had inferred 30 years earlier, in reception, not the generation of the polarising region signal. But why would loss of Shh signal perception cause polydactyly rather than a loss of digits as in *Shh* mutant mouse embryos? The answer lay in a small organelle, the primary cilium, which projects from the surface of most cell types in the developing embryo and adult. After a decade of analysis, we now know that *Ptch1* and *Smo* are localised to primary cilia during different phases of activation of the Shh pathway - *Ptch1* during Shh signalling, *Smo* when there is no Shh signalling - and, furthermore, that the Gli transcription factors have to be trafficked through the cilia, where they are modified to allow them to enter the nucleus to repress or activate expression of Shh target genes (reviewed Bangs and Anderson, 2017).

Cilia are increasingly associated with a class of human congenital diseases - the 'ciliopathies' which are due to loss of cilia or impaired cilia function. They include organ specific ciliopathies such as

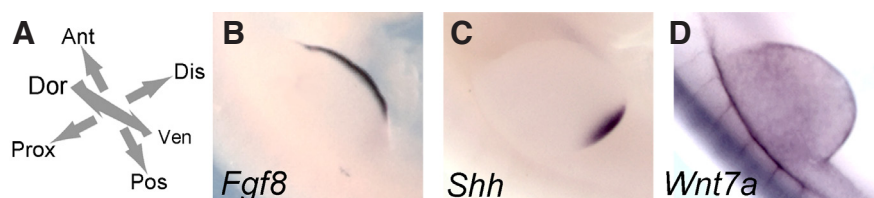


Fig. 4. Expression patterns of genes encoding important signalling molecules in early chick wing bud. (A) Three main axes. **(B)** *Fgf8* in apical ectodermal ridge rimming distal tip. **(C)** *Shh* in polarizing region at posterior margin. **(D)** *Wnt7a* in dorsal ectoderm. Prox, proximal; Dis, distal; Dor, dorsal; Ant, anterior; Pos, posterior; Ven, ventral.

polycystic kidney disease and retinopathies, and syndromes such as Biedel- Bardot syndrome, Orofacial -digital syndrome (OFD), and Short-rib polydactyly which frequently include polydactyly. In 2006, *talpid³* was mapped in to a novel gene- *KIAA0586* (Davey *et al.*, 2006). TALPID3/KIAA0586 was subsequently shown to be a centrosomal protein, the loss of which causes absence of all cilia (both primary and motile cilia) not only in chicken (Yin *et al.*, 2009), but also mouse and zebrafish (reviewed Bangs and Anderson, 2017). *Talpid³* was later shown to encode a known centrosomal protein C2CD3 (Chang *et al.*, 2014). Both TALPID3/KIAA0586 and C2CD3 proteins localise to the basal body- a modified centriole which forms the base of a cilium. In the case of TALPID3/KIAA0586, loss of the protein causes failure of the centriole to migrate to the cell surface and dock with the plasma membrane (Stephen *et al.*, 2014). As almost all cilia are lost, Shh signalling via the Gli transcription factors is neither fully activated nor repressed. The function of C2CD3 is still elusive but the number of cilia is severely reduced, compromising Shh signalling.

The study of these chicken mutants has spearheaded novel advances because they have been informative about both mechanisms involved in ciliogenesis and human disease loci as patients with *TALPID3* and *C2CD3* gene mutations have been identified (reviewed Bangs and Anderson, 2017). *TALPID3* mutations in human are usually recessive and cause a range of phenotypes from early embryonic lethality with polydactyly and craniofacial defects through to syndromes such as Jeune and Joubert syndromes which affect other parts of the body. Mutations in *C2CD3* were already known to cause an OFD-type ciliopathy characterised by polysyndactyly and other abnormalities.

Molecular basis of antero-posterior positional values

One response to polarizing region signalling at the anterior margin of a chick wing bud is mirror-image duplication in gene expression patterns across the antero-posterior axis, including expression of 5' genes belonging to *Hoxa* and *Hoxd* clusters. The nested expression of *Hoxd* genes, centred on the posterior-distal region of the early limb bud in the first phase of expression suggested that they encode antero-posterior position. Furthermore, in *talpid* limb buds, *Hoxd* genes are instead expressed throughout the antero-posterior axis correlating with loss of digit identity. When *Hoxd11* was overexpressed in chick limbs using retroviruses- the first time this retroviral technique was used in the limb - an additional digit developed (Morgan *et al.*, 1992). It is likely however in the light of subsequent work in the mouse that this was due to inducing ectopic *Shh* expression.

Other molecules that could encode antero-posterior positional information acting downstream of Shh in the wing bud include the T-box transcription factors, *Tbx2/3*, expressed in distinct anterior and posterior stripes and the transcription factors, *Sal1* and *Sal3*, also expressed in regions fated to become digits (Fisher *et al.*, 2011). Overexpression of *Tbx2/3* in the developing chick leg by retroviral vectors, resulted in additional phalanx formation and apparent posterior transformations in digit identity (Suzuki *et al.*, 2004). These families of transcription factors are clinically relevant, for example, patients with a *SALL4* mutation, have limb reductions. Many other conserved gene targets of Shh signalling have been identified following genomic screens in chick and mouse limbs (Bangs *et al.*, 2010, Vokes *et al.*, 2008), including BMPs, implicated as secondary signals acting downstream of Shh

signalling and specifying antero-posterior positional values in the chick wing. Further targets of Shh signalling regulate proliferation and include *N-myc* and *Cyclins D1* and *D2* – explaining the direct effect of polarizing region grafts on wing bud growth.

BMPs are produced by the interdigital regions between the digit condensations and manipulations of BMP signalling at these stages alter digit morphology accounting for the effects of grafting interdigital mesenchyme (Suzuki *et al.*, 2008). Interestingly, SMAD activity, a read-out of the response to BMP signalling, is graded between adjacent digit condensations, mirroring earlier graded Shh signalling, thus providing a connection between specification of positional information and later interpretation (eg: Vargesson and Laufer, 2009). To define the molecular basis of the identity of the digit condensations -and gain insights into homologies, the transcriptomes for each digit condensation in the chick wing and chick leg were analysed but revealed both similarities and differences (Wang *et al.*, 2011).

Molecules mediating dorso-ventral patterning

Significant progress has been made in identifying molecules produced by the ectoderm that specify dorso-ventral pattern (reviewed Tickle and Towers, 2009). *Wnt7a* is expressed by the dorsal ectoderm of both chick and mouse limb buds and genetic deletion of *Wnt7a* in the mouse demonstrated its role in dorsal patterning producing “double --ventral” digits. A good candidate for a factor encoding dorsal positional information is the transcription factor *Lmx1b*. *Lmx1b* is expressed by the dorsal mesenchyme of the chick wing and acts downstream of *Wnt7a*. Retroviral over-expression of *Lmx1b* in the chick wing resulted in ventral to dorsal transformations of the mesenchyme (Riddle *et al.*, 1995; Vogel *et al.*, 1995) similar to the “double -dorsal” wings induced by grafting an apical ridge to the dorsal side of a wing bud. Further analyses in the mouse revealed that loss of *Lmx1b* results in a “double -ventral” phenotype - in which nails fail to form on either side of the digits. *LMBX1* is the gene responsible for nail-patella syndrome which affects development of these dorsal structures.

Experiments in chick wing buds showed that BMP signals produced by the ventral ectoderm specify ventral pattern (Pizette *et al.*, 2001). Genes acting downstream of BMP signalling in the ectoderm include *Engrailed 1*, and its inactivation in the mouse limb results in a “double-dorsal” phenotype. Experiments on the chick wing showed that over-expression of *Engrailed-1* results in loss of *Wnt7a* expression in dorsal ectoderm (Logan *et al.*, 1997). Therefore, *Engrailed-1* determines ventral fate by repressing expression of the dorsalizing factor gene *Wnt7a*.

Limbs of *Wnt7a*^{-/-} mice lack posterior digits, found to be due to reduced *Shh* expression at the posterior limb bud margin. Additional work on the chick wing, removing the dorsal ectoderm surgically, also revealed that *Wnt7a* regulates *Shh* expression (Yang *et al.*, 1995). The importance of this effect of Wnt signalling is that it integrates patterning along antero-posterior and dorso-ventral axes.

Conclusions

We have highlighted how studies of chick embryos elucidated the basic biology of limb development and helped uncover its genetic basis. We have not attempted to be comprehensive – transcriptome analysis estimates that about 10,000 genes are expressed in the chick wing bud! (Boardman *et al.*, 2003)- but underscored

genes for signalling molecules and transcription factors with crucial functions particularly those of clinical relevance. Future progress towards obtaining a more complete picture will include applying more genomic level approaches. We anticipate that the chick limb will feature in such endeavours and complement studies on mammalian limbs. Successes in delivering the gene editing CRISPR/ Cas9 system to chick embryos by electroporation have been reported (e.g. Veron *et al.*, 2015) and this could provide an important new tool for future studies.

Throughout this review, we featured chicken mutants - in particular, their contributions to identifying new genes involved in limb development - also studies on teratogenesis using chick embryos. With respect to chicken mutants, there is considerable scope for further advances. The genes affected in the *limbless* / *wingless* mutant strains are currently unknown as are those in other avian mutants such as the quail mutant *hereditary multiple malformations* and the *diplopodia* chicken mutants (*dp¹*, *dp³*, *dp⁴*; Fig. 3) which may or may not be ciliopathies and the chicken *eudiplopodia* mutant which has multiple apical ridges. Uncovering the genes affected, just as in the chicken *talpid* mutants, may give unexpected insights into human disease (Robb *et al.*, 2011). With respect to teratogenicity, the developing chick limb is currently being used to test drugs such as Valproate (Whitsel *et al.*, 2002) as well screening thalidomide analogs to identify those clinically beneficial but not teratogenic (Beedie *et al.*, 2016b). This screening is necessary because thalidomide is being widely used in parts of Brazil to treat complications of leprosy and tragically a new generation of thalidomide babies has been born (Vargesson, 2015). Finally, the studies on thalidomide spotlighted the limb vasculature. Intriguingly, the vasculature of *talpid³* limb buds is abnormal with accompanying changes in expression of angiogenic signalling molecules (Davey *et al.*, 2007). This suggests that focussing on the development of the limb vasculature would be a very valuable area for future research and an area in which chick embryos could make important contributions.

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References

- ABBOTT UK, TAYLOR LW, ABPLANALP H (1960). Studies with *talpid²*, an embryonic lethal of the fowl. *J Hered* 51: 195-202.
- ALTABEF M, CLARKE JDW, TICKLE C (1997). Dorso-ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. *Development* 124: 4547-4556.
- AMBLER CA, NOWICKI JL, BURKE AC *et al.* (2001). Assembly of trunk and limb blood vessels involves extensive migration and vasculogenesis of somite-derived angioblasts. *Dev Biol* 234: 352-364.
- BANGS F, WELTEN M, DAVEY MG *et al.* (2010). Identification of genes downstream of the Shh signalling in the developing chick wing and syn-expressed with Hoxd13 using microarray and 3D computational analysis. *Mech Dev* 127: 428-441.
- BANGS F, ANDERSON KV (2017). Primary Cilia and Mammalian Hedgehog Signaling. *Cold Spring Harb Perspect Biol* pii: a028175. doi: 10.1101/cshperspect.a028175.
- BEEIDIE SL, MAHONY C, WALKER HM, *et al.* (2016a). Shared mechanism of teratogenicity of anti-angiogenic drugs identified in the chicken embryo model. *Sci. Rep.* 6: 30038.
- BEEIDIE SL, RORE HM, BARNETT S *et al.* (2016b). *In vivo* screening and discovery of novel candidate thalidomide analogues in the zebrafish embryo and chicken embryo model systems. *Oncotarget* 7: 33237-33245.
- BOARDMAN PE, SANZ-EZQUERRO J, OVERTON IM *et al.* (2002). A comprehensive collection of chicken cDNAs. *Curr Biol* 12: 1965-1969.
- BOYLEN JB, HORNE HH, JOHNSON WJ (1963). Teratogenic effects of thalidomide and related substances. *Lancet* 1: 552.
- CARRINGTON JL, FALLON JF (1984). Evidence that the ectoderm is the affected germ layer in the wingless mutant chick embryo. *J Exp Zool* 232: 297-308.
- CHANG CF, SCHOCK EN, O'HARE EA *et al.* (2014). The cellular and molecular etiology of the cranial defects in the avian ciliopathic mutant *talpid²*. *Development* 141: 3003-3012.
- CHINNAIYAK, TICKLE C, TOWERS M (2014). Sonic hedgehog-expressing cells in the developing limb measure time by an intrinsic cell cycle clock. *Nat Commun* 5: 4230.
- CHRIST B, JACOB HJ, JACOB M (1977). Experimental analysis of the origin of the wing musculature in avian embryos. *Anat Embryol (Berl)* 150: 171-186.
- COHN M, IZPISUA-BELMONTE JC, ABUD H *et al.* (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* 80: 239-246.
- COOPER KL, HU JK, TEN BERGE D *et al.* (2011). Initiation of proximo distal patterning in the vertebrate limb by signals and growth. *Science* 332: 1083-1086.
- DAHAN RD, FALLON JF (2000). Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. *Science* 289: 438-441.
- D'AMATO RJ, LOUGHNAN MS, FLYNN E, *et al.* (1994). Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* 91: 4082-4085.
- DAVEY MG, PATON IR, YIN Y *et al.* (2006). The chicken *talpid³* gene encodes a novel protein essential for Hedgehog signaling. *Genes Dev* 20: 1365-1377.
- DAVEY MG, JAMES J, PATON IR *et al.* (2007). Analysis of *talpid³* and wild-type chicken embryos reveals roles for Hedgehog signalling in development of the limb bud vasculature. *Dev Biol* 301: 155-165.
- D'SOUZA D, McDIARMID J, TICKLE C (1998). A polydactylous human foot with 'double-dorsal' toes. *J Anat* 193: 121-130.
- DUDLEY AT, ROS MA, TABIN CJ (2002). Are-examination of proximodistal patterning during vertebrate limb development. *Nature* 418: 539-544.
- DUNN IC, PATON IR, CLELLAND AK *et al.* (2011). The chicken *Polydactyly (Po)* locus causes allelic imbalance and ectopic expression of Shh during limb development. *Dev Dyn* 240: 1163-1172.
- EDE DA, KELLY WA (1964). Developmental abnormalities in the trunk and limbs of the *talpid³* mutant of the fowl. *J Embryol Exp Morphol* 12: 339-356.
- EDE DA, SHAMSLAHIDJANI M (1983). Ectoderm/mesoderm recombination, dissociation and cell aggregation studies in normal and talpids mutant avian embryos. *Prog Clin Biol Res* 110 Pt A: 45-55.
- EICHELE G, TICKLE C, ALBERTS BM (1984). Microcontrolled release of biologically active compounds in chick embryos: beads of 200-microns diameter for the local release of retinoids. *Anal Biochem* 142: 542-555.
- FALLON JF and CROSBY GM (1977). Polarizing zone activity in limb buds of amniotes. In *Limb and Somite Morphogenesis* (Ed. D.A. Ede, J.R. Hinchliffe, M. Balls). Cambridge University Press, Cambridge, pp. 55-71.
- FISHER M, DOWNIE H, WELTEN MC *et al.* (2011). Comparative analysis of 3D expression patterns of transcription factor genes and digit fate maps in the developing chick wing. *PLoS One* 6: e18661.
- GREEN JB, SHARPE J (2015). Positional information and reaction-diffusion: two big ideas in developmental biology combine. *Development* 142: 1203-1211.
- GROS J, TABIN CJ (2014). Vertebrate limb bud formation is initiated by localized epithelial-to-mesenchymal transition. *Science* 343: 1253-1256.
- GALLOWAY JL, DELGADO I, ROS MA *et al.* (2009). A reevaluation of X-irradiation-induced phocomelia and proximodistal limb patterning. *Nature* 460: 400-404.
- HILL RE, LETTICE LA (2013). Alterations to the remote control of Shh gene expression cause congenital abnormalities. *Philos Trans R Soc Lond B* 368: 20120357.
- HINCHLIFFE JR, EDE DA (1973). Cell death and the development of limb form and skeletal pattern in normal and *wingless (ws)* chick embryos. *J Embryol Exp Morphol* 30: 753-772.
- JOHNSON EJ, NEELY DM, DUNN IC *et al.* (2014). Direct functional consequences

- of ZRS enhancer mutation combine with secondary long range SHH signalling effects to cause preaxial polydactyly. *Dev Biol* 392: 209-220.
- JURAND A (1966). Early changes in limb buds of chick embryos after thalidomide treatment. *J Embryol Exp Morphol* 16: 289-300.
- KAWAKAMI Y, CAPDEVILA J, BUSCHER D *et al.* (2001). WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* 104: 891-900.
- LAUFER E, NELSON CE, JOHNSON RL *et al.* (1994). Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 79: 993-1003.
- LEWIS KE, DROSSOPOULOU G, PATON IR *et al.* (1999). Expression of ptc and gli genes in *talpid³* suggests bifurcation in Shh pathway. *Development* 126: 2397-2407.
- LOGAN C, HORNBRUCH A, CAMPBELL I *et al.* (1997). The role of Engrailed in establishing the dorsoventral axis of the chick limb. *Development* 124: 2317-2324.
- MAAS SA, SUZUKI T, FALLON JF (2012). Identification of spontaneous mutations within the long-range limb-specific Sonic hedgehog enhancer (ZRS) that alter Sonic hedgehog expression in the chicken limb mutants oligozeugodactyly and silkie breed. *Dev Dyn* 240: 1212-1222.
- MAHONY C, McMENEMY S, RAFIPAY AJ *et al.* (In Press). CPS49-induced neurotoxicity does not cause limb patterning anomalies in developing chicken embryos. *J Anat* doi: dx.doi.org/10.1111/joa.12712
- MAHONY C, VARGESSON N (2013). Molecular analysis of regulative events in the developing chick limb. *J Anat* 223: 1-13.
- MCCREDIE J, MCBRIDE WG (1973). Some congenital abnormalities: possibly due to embryonic peripheral neuropathy. *Clin Radiol* 24: 204-211.
- MERCADER N, LEONARDO E, PIEDRA ME, *et al.* (2000) Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of Meis genes. *Development* 127: 3961-3970
- MICHAUD JL, LAPOINTE F, Le DOUARIN NM (1997). The dorsoventral polarity of the presumptive limb is determined by signals produced by the somites and by the lateral somatopleure. *Development* 124: 1453-1463.
- MONTAVON T, DUBOULE D (2013). Chromatin organization and global regulation of Hox gene clusters. *Philos Trans R Soc Lond B* 368: 20120367.
- MORGAN BA, IZPISÚA-BELMONTE JC, DUBOULE D *et al.* (1992). Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. *Nature* 358: 236-239.
- NELSON CE, MORGAN BA, BURKE AC *et al.* (1996). Analysis of Hox gene expression in the chick limb bud. *Development* 122: 1449-1466.
- NISHIMOTO S, LOGAN MP (2016). Subdivision of the lateral plate mesoderm and specification of the forelimb and hindlimb forming domains. *Semin Cell Dev Biol* 49: 102-108.
- NISWANDER L, TICKLE C, VOGEL A *et al.* (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* 75: 579-587.
- NISWANDER L, JEFFREY S, MARTIN GR *et al.* (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371: 609-612.
- OHUCHI H, SHIBUSAWA M, NAKAGAWA T *et al.* (1997a). A chick *wingless* mutation causes abnormality in maintenance of Fgf8 expression in the wing apical ridge, resulting in loss of the dorsoventral boundary. *Mech Dev* 62: 3-13.
- OHUCHI H, NAGAWA T, YAMAMOTO A *et al.* (1997b). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* 124: 2235-2244.
- PEARSE RV, SCHERZ PJ, CAMPBELL JK *et al.* (2007). A cellular lineage analysis of the chick limb bud. *Dev Biol* 310: 388-400.
- PICKERING J, TOWERS M (2016). Inhibition of Shh signalling in the chick wing gives insights into digit patterning and evolution. *Development* 143: 3514-3521.
- PIZETTE S, ABATE-SHEN C, NISWANDER L (2001). BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. *Development* 128: 4463-4474.
- RIDDLE RD, JOHNSON RL, LAUFER E *et al.* (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75: 1401-1416.
- RIDDLE RD, ENSINI M, NELSON CE *et al.* (1995). Induction of the LIM homeobox gene *Lmx1* by *Wnt-7a* establishes dorsoventral pattern in the vertebrate limb. *Cell* 83: 631-640.
- ROBB EA, GITTER CL, CHENG HH *et al.* (2011). Chromosomal mapping and candidate gene discovery of chicken developmental mutants and genome-wide variation analysis of MHC congenics. *J Hered* 102: 141-156.
- ROMANOFF AL (1972). Pathogenesis of the avian embryo. An analysis of causes of malformations and prenatal death. Wiley, New York. 464 pp.
- ROSMA, DAHN RD, FERNANDEZ-TERAN M *et al.* (2003). The chick oligozeugodactyly (ozd) mutant lacks sonic hedgehog function in the limb. *Development* 130: 527-537.
- ROSELLO-DIEZ A, ROS MA, TORRES M (2011). Diffusible signals not autonomous mechanisms determine the main proximodistal limb subdivision. *Science* 332: 1086-1088.
- SABHERWAL N, BANGS F, ROTH R *et al.* (2007). Long-range conserved non-coding SHOX sequences regulate expression in developing chicken limb and are associated with short stature phenotypes in human patients. *Hum Mol Genet* 16: 210-222.
- SAIZ-LOPZ P, CHINNAIYA K, CAMPA VM *et al.* (2015). An intrinsic timer specifies distal structures of the vertebrate limb. *Nat Commun* 6: 8108.
- SAIZ-LOPZ P, CHINNAIYA K, TOWERS M *et al.* (2017). Intrinsic properties of limb bud cells can be differentially reset. *Development* 144: 479-486.
- SANDERS TA, LLAGOSTERA E, BARNAM (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. *Nature* 497: 628-632.
- SANZ-EZQUERRO JJ, TICKLE C (2003). Fgf signaling controls the number of phalanges and tip formation in developing digits. *Curr Biol* 13: 1830-1836.
- SIAMWALAJH, VEERIAH V, PRIYAMK, *et al.* (2012). Nitric oxide rescues thalidomide mediated teratogenicity. *Sci Rep* 2: 679.
- SAUNDERS JW (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J Exp Zool* 108: 363-403.
- SAUNDERS JW, GASSELING MT (1968). Ectodermal-mesenchymal interactions in the origin of limb symmetry. In *Mesenchymal-epithelial Interactions* (Eds. R. Fleischmeyer and R. E. Billingham). Williams and Wilkins, Baltimore, pp. 78-97.
- SAUNDERS JW (1977). The experimental analysis of chick limb development. In *Limb and Somite Morphogenesis* (Eds. D.A. Ede, J.R. Hinchliffe, M. Balls). Cambridge University Press, Cambridge, pp. 1-24.
- SHELLSWELL GB and WOLPERT L (1977). The pattern of muscle and tendon development in the chick wing. In *Limb and Somite Morphogenesis* (Eds. D.A. Ede, J.R. Hinchliffe, M. Balls). Cambridge University Press, Cambridge, pp. 71-86.
- SMYTH JR, SREEKUMAR GP, COYLE CA *et al.* (2000). A new recessive *ametapodia* mutation in the chicken (*Gallus domesticus*). *J Hered* 91: 340-342.
- STEPHEN LA, TAWAMIE H, DAVIS GM *et al.* (2015). TALPID3 controls centrosome and cell polarity and the human ortholog KIAA0586 is mutated in Joubert syndrome (JBTS23). *Elife* 4. pii: e08077.
- STRECKER TR, STEPHENS TD (1983). Peripheral nerves do not play a trophic role in limb skeletal morphogenesis. *Teratology* 27: 159-167.
- SUMMERBELL D, LEWIS JH, WOLPERT L (1973). Positional information in chick limb morphogenesis. *Nature* 244: 492-496.
- SUMMERBELL D (1974). A quantitative analysis of the effect of excision of the AER from the chick limb-bud. *J Embryol Exp Morphol* 32: 651-660.
- SUZUKI T, TAKEUCHI J, KOSHIBA-TAKEUCHI K *et al.* (2004). *Tbx* genes specify posterior digit identity through Shh and BMP signaling. *Dev Cell* 6: 43-53.
- SUZUKI T, HASSO SM, FALLON JF (2008) Unique SMAD1/5/8 activity at the phalanx-forming region determines digit identity. *Proc Natl Acad Sci USA* 105: 4185-4190.
- TABINCJ (1998). A developmental model for thalidomide defects. *Nature* 396: 322-323.
- THERAPONTOS C, ERSKINE L, GARDNER E, *et al.* (2009). Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. *Proc Natl Acad Sci USA* 106: 8573-8578.
- TICKLE C, ALBERTS B, WOLPERT L *et al.* (1982). Local application of retinoic acid to the limb bud mimics the action of the polarizing region. *Nature* 296: 564-565.
- TICKLE C, EICHELE G (1994). Vertebrate limb development. *Ann Rev Cell Biol* 10, 121-152.
- TICKLE C (2015). How the embryo makes a limb: determination, polarity and identity. *J Anat* 227: 418-430.
- TICKLE C (2017). An historical perspective on the pioneering experiments of John Saunders. *Dev Biol* (dx.doi.org/10.1016/j.ydbio.2017.05.028).
- TICKLE C, TOWERS M (2017). Sonic Hedgehog Signaling in Limb Development. *Front Cell Dev Biol* 5: 14.
- TIECKE E, BANGS F, BLASCHKE R *et al.* (2006). Expression of the short stature homeobox gene *Shox* is restricted by proximal and distal signals in chick limb

- buds and affects the length of skeletal elements. *Dev Biol* 298: 585-596.
- TOWERS M, MAHOOD R, YIN Y *et al.* (2008). Integration of growth and specification in chick wing digit-patterning. *Nature* 452: 882-886.
- TOWERS M, TICKLE C (2009). Growing models of vertebrate limb development. *Development* 136: 179-190.
- TOWERS M, SIGNOLET J, SHERMAN A *et al.* (2011). Insights into bird wing evolution and digit specification from polarizing region fate maps. *Nat Commun* 2: 426.
- VARGESSON N (2013). Thalidomide Embryopathy: an enigmatic challenge. *ISRN Dev. Biol.* (<http://dx.doi.org/10.1155/2013/241016>).
- VARGESSON N (2015). Thalidomide-induced teratogenesis: History and mechanisms. *Birth Defects Research (Part C)* 105: 140-156.
- VARGESSON N, LAUFER E (2001). Smad7 misexpression during embryonic angiogenesis causes vascular dilation and malformations independently of vascular smooth muscle cell function. *Dev Biol* 240: 499-516.
- VARGESSON N, LAUFER E (2009). Negative Smad expression and regulation in the developing chick limb. *PLoS One* 4: e5173.
- VERON N, QU Z, KIPEN PA *et al.* (2015). CRISPR mediated somatic cell genome engineering in the chicken. *Dev Biol* 407: 68-74.
- VOGELA, RODRIGUEZ C, WARNKEN W *et al.* (1995). Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature* 378: 716-720.
- VOKES SA, JI H, WONG WH *et al.* (2008). A genome-scale analysis of the cis-regulatory circuitry underlying Shh-mediated patterning of the mammalian limb. *Genes Dev* 22: 2651-2663.
- WANG Z, YOUNG RL, XUE H *et al.* (2011). Transcriptomic analysis of avian digits reveals conserved and derived digit identities in birds. *Nature* 477: 583-586.
- WELLIK DM, CAPECCHI MR (2003). Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. *Science* 301: 363-367.
- WHITSEL AI, JOHNSON CB, FOREHAND CJ (2002). An *in ovo* chicken model to study the systemic and localized teratogenic effects of valproic acid. *Teratology* 66: 153-163.
- WILBY O, EDE DA (1975). A model generating the pattern of cartilage skeletal elements in the embryonic chick limb. *J Theor Biol* 52: 199-217.
- WOLPERT L (1969). Positional information and the spatial pattern of cellular differentiation. *J Theor Biol* 25: 1-47.
- WOLPERT L (1989). Positional information revisited. *Development* 107: 3-12.
- WOLPERT L (2018). From soil mechanics to chick development. *Int J Dev Biol*. 62: (dx.doi.org/10.1387/ijdb.180030-LW).
- WOLPERT L, TICKLE C, SAMPFORD M (1979). The effect of cell killing by X-irradiation on pattern formation in the chick limb. *J Embryol Exp Morph* 50: 175-193.
- WOOLLEY TE, BAKER RE, TICKLE C *et al.* (2014). Mathematical modelling of digit specification by a sonic hedgehog gradient. *Dev Dyn* 243: 290-298.
- YANG Y, NISWANDER L (1995). Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 80: 939-947.
- YIN Y, BANGS F, PATON IR *et al.* (2009). The *Talpid*^β gene (*KIAA0586*) encodes a centrosomal protein that is essential for primary cilia formation. *Development* 136: 655-664.
- ZENG X, GOETZ JA, SUBER *et al.* (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. *Nature* 411: 716-720.
- ZHANG Z, NIE C, JIA Y *et al.* (2016). Parallel Evolution of Polydactyly Traits in Chinese and European Chickens. *PLoS One*. 11: e0149010.
- ZUNIGAA, ZELLER R, PROBST S (2012). The molecular basis of human congenital limb malformations. *Wiley Interdiscip Rev Dev Biol* 1: 803-822.
- ZWILLING E (1974). Effects of contact between mutant *wingless* limb buds and those of genetically normal chick embryos: confirmation of a hypothesis. *Dev Biol* 39: 37-48.

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